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Factor H Binding to Bone Sialoprotein and Osteopontin Enables Tumor Cell Evasion of Complement-mediated Attack*

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Metastatic cancer cells, like trophoblasts of the developing placenta, are invasive and must escape immune surveillance to survive. Complement has long been thought to play a significant role in the tumor surveillance mechanism. Bone sialoprotein (BSP) and osteopontin (OPN, ETA-1) are expressed by trophoblasts and are strongly up-regulated by many tumors. Indeed, BSP has been shown to be a positive indicator of the invasive potential of some tumors. In this report, we show that BSP and OPN form rapid and tight complexes with complement Factor H. Besides its key role in regulating complement-mediated cell lysis, Factor H also appears to play a role when "hijacked" by invading organisms in enabling cellular evasion of complement. We have investigated whether BSP and OPN may play a similar role in tumor cell complement evasion by testing to see whether these glycoproteins could promote tumor cell survival. Recombinant OPN and BSP can protect murine erythroleukemia cells from attack by human complement as well as human MCF-7 breast cancer cells and U-266 myeloma cells from attack by guinea pig complement. The mechanism of this gain of function by tumor cell expression of BSP or OPN has been defined using specific peptides and antibodies to block BSP and OPN protective activity. The expression of BSP and OPN in tumor cells provides a selective advantage for survival via initial binding to $\alpha_V\beta_3$ integrin (both) or CD44 (OPN) on the cell surface, followed by sequestration of Factor H to the cell surface and inhibition of complement-mediated cell lysis.

Osteopontin (OPN)[1] and bone sialoprotein (BSP) are produced by trophoblasts (1, 2) and are induced in certain neoplasms (3–11). BSP is a phosphoprotein of molecular mass ~70–80 kDa, about half of which is sialic acid rich *N*- and *O*-linked carbohydrates, and also contains several glutamic acid-rich domains, tyrosine sulfates, as well as an integrin-binding arginine-glycine-aspartate (RGD) domain (12–14). During normal human development, BSP is produced by cells of the skeleton (osteoblasts, osteoclasts, osteocytes, and hypertrophic chondrocytes) as well as trophoblasts (2). Because of its primary association with cells that produce a mineralized extracellular matrix, BSP has been hypothesized to play a role in mineralization where its high degree of negative charge could function in calcium sequestration or in hydroxyapatite crystal nucleation. However, the absence of a clear skeletal phenotype in the BSP knock-out mouse suggests either the existence of molecular redundancy or another as yet undefined functional role for BSP. Because of its apparent restricted expression pattern in trophoblasts and skeletal cells, BSP expression in tumors has been proposed to play a role in either microcalcification (15–17) or in metastasis homing to bone (3, 9, 18). The level of BSP expression correlates positively with disease severity (9, 18, 19).

The second protein, OPN, is also known as Secreted PhosphoProtein I, 2ar, early T-lymphocyte activation 1, and transformation-associated phosphoprotein. It is a protein of ~60 kDa, and shares with BSP high sialic acid content, highly acidic sequences (but these are aspartic acid-rich), multiple residues with consensus for phosphorylation as well as an integrin-binding RGD motif (6, 12, 14). Because of its RGD tripeptide and adhesive properties, it has been proposed that OPN plays a role in metastasis in certain tumors (20–22). OPN expression is associated with clinical severity in lung cancer (20), lymph node negative breast cancer (23), gastric cancer (24), and perhaps ovarian carcinoma (25). In light of their induction by certain neoplasms, we investigated the possible role of these proteins in one aspect of escaping host humoral surveillance.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit anti-human BSP antibodies LF-83, LF-100, LF-119, LF-120, and LF-125 have been previously described (26). Rabbit anti-BSP peptide-derived antibody LF-142 and a mouse monoclonal antibody LFmAb-11 were raised against the sequence EY*EY*TGVNEY*DNGY*EIY*ESENGEP (amino acids 258–285) conjugated to horseshoe crab hemocyanin, where the Y* denotes tyrosine sulfates. Normal human serum, purified human complement Factor H protein, and mouse monoclonal antibody against Factor H were obtained from Quidel Corp. (San Diego, CA). Polyclonal antibodies against CD-44 and a "functional" antibody against $\alpha_V\beta_3$ were obtained from Chemicon Co. (Temecula, CA). Synthetic purified glycine-arginine-aspartate-serine peptide (GRGDS) was obtained from Calbiochem-Nova-Biochem Corp. (La Jolla, CA). Preimmune serum, human serum adsorbed goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) as well as goat anti-mouse conjugated to HRP were obtained from Kirkegaard & Perry (Gaithersburg, MD). HRP-conjugated strepavidin and sulfosuccinimido-biotin were obtained from Pierce Chemical Co. (Chicago, IL). α-Minimal essential medium (α-MEM), Dulbecco's modified essential medium (DMEM), RPMI 1640, Eagle's minimal essential medium (EMEM), Earle's balanced salt solution, Hank's balanced salt solution, and heat inactivated fetal bovine serum were obtained from BioFluids, Inc. (Rockville, MD).

Western Blotting—Samples diluted in gel sample buffer were resolved by SDS-PAGE 4–20% gradient gels (Novex Corp, San Diego, CA), transferred to nitrocellulose following standard conditions (27).

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[1] The abbreviations used are: OPN, osteopontin; BSP, bone sialoprotein; HRP, horseradish peroxidase; TBS, Tris-buffered saline; MEL, murine erythroleukemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine; SEC, size exclusion chromatography; MEM, minimal essential medium; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; ACP, alternate complement pathway.

Nitrocellulose membranes were rinsed with Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-Tween). After a 1-h incubation in blocking solution (TBS-Tween + 5% non-fat powdered milk) at room temperature on rotary shaker, primary antibody was added and incubated overnight at 4 °C. The nitrocellulose sheet was washed in TBS-Tween four times for 5 min each time with TBS-Tween and then second antibody in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. Following removal of the second antibody solution the membrane was washed three times with TBS-Tween and rinsed a final time in enzyme substrate buffer for 5 min. Enhanced chemiluminescence reagents were employed for signal detection (Pierce) with x-ray film.

High Performance Liquid Chromatography—A Shimadzu LC10AS binary gradient system was employed for chromatographic separations. A 1.0-ml packed volume ToyoPearl QAE (TosoHaas, Montgomeryville, PA) column was pre-equilibrated with 0.05 M sodium phosphate, pH 7.4, containing 50% fresh formamide. A linear salt gradient increasing to 2.0 M NaCl at 2.0 ml/min flow rate over 50 min was employed collecting 1-min fractions. Size exclusion chromatography utilized a 1.0 × 30-cm Superose 6 column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 0.05 M sodium phosphate, pH 7.4, containing 50% fresh formamide at a flow rate of 0.5 ml/min. The column was calibrated using commercially available protein standards of known molecular weight (Amersham Pharmacia Biotech).

Direct ELISA—Greiner high-binding 96-well plates were coated with 100-μl HPLC fractions overnight at 4 °C. Plates were washed three times (5 min each) with TBS-Tween and exposed to 100 μl of 1:2000 primary antibody for 1 h at room temperature. Plates were washed three times and exposed to 100 μl of 1:2000 HRP-conjugated goat anti-rabbit IgG. Following a 1-h incubation at room temperature, plates were washed again three times with TBS-Tween and color was developed using 3,3′,5,5′-tetramethylbenzidine and H_2O_2 for ≤10 min at room temperature. Color development was stopped by the addition of 25 μl of 1 N H_2SO_4 and analyzed at 450 nm.

Immunoprecipitation—Aliquots of normal human serum diluted 1:100 in immunoprecipitation buffer (0.1 M Tris, pH 7.2, 0.15 M NaCl, .05% Tween 20, and 1% aprotinin) were incubated sequentially with 0.1 ml each of (*a*) Protein G-agarose (Kirkegaard & Perry); (*b*) normal rabbit serum IgGs bound to Protein G-agarose; (*c*) rabbit anti-BSP antibodies bound to Protein G-agarose. Each incubation for 1 h at 4 °C was terminated by centrifugation at 10,000 × *g* for 5 min and the supernatant taken to the next immunoprecipitation. The first two incubations removed proteins binding nonspecifically to agarose and to normal rabbit serum. The anti-BSP incubation immunoprecipitate was dissolved in gel sample buffer and analyzed by 4–20% gradient SDS-PAGE.

Production of Recombinant BSP (rBSP) and OPN (rOPN)—Adenoviral constructs were generated by subcloning BSP (13), BSP-KAE or OPN (28) cDNA into high expression, replication-deficient adenovirus (Ad5) using EF-1 (BSP) and CMV (BSP-KAE, OPN) promoters, respectively. The RGD→KAE constructs were made using *in situ* mutagenesis and the entire insert checked for fidelity. Adenoviruses were plaque-selected and propagated on HEK 293 cells (ATCC number CRL1573). Cells were harvested when cytopathic effects were present and lysed by 5 freeze-thaw cycles. Cellular debris was removed and viral particles were purified by twice banding on CsCl. After dialysis in Tris/$MgCl_2$/glycerol buffer at 4 °C, viruses were aliquoted and frozen at −70 °C. Evaluation of viral titers was carried out by plaque formation of virus dilutions on HEK293 cells (29). Typically ~2–4 × 10^{11} plaque forming units/ml were obtained from one viral preparation. Recombinant proteins were generated by infecting subconfluent normal human marrow stromal fibroblasts with 10,000 plaque forming units/cell. Cells were maintained in α-MEM, 20% fetal bovine serum, and 100 IU/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Medium was changed to serum-free conditions after 48 h. Subsequently, medium was collected every 24 h and frozen at −70 °C. Aliquots were assayed by SDS-gel electrophoresis and Western blot for BSP and OPN expression. Expression was found to be at highest levels at ~168 h post-infection. The proteins were purified by routine column chromatography. Native BSP, BSP-KAE, and OPN proteins were purified by diluting medium from normal human marrow stromal fibroblast cells 1:1 with 40 mM phosphate buffer, pH 7.4, and loading directly on a 5.0 × 2.0-cm column packed with ToyoPearl TSK QAE resin. A linear salt gradient to 2.0 M NaCl was employed to purify the BSP and OPN to ~95% purity as measured by SDS-PAGE.

Biotinylated BSP—12 μg of recombinant human BSP was dissolved in 50 μl of PBS. 2 μl of a fresh 1 mg/ml solution of NHS-LC-Biotin (Pierce) was added and the reaction incubated at room temperature for 45 min. The unreacted biotin was removed by repeated washing with TBS-Tween and centrifugation in a Microcon 30 (Amicon, Beverly, MA). A final volume of 50 μl was retained.

Alternate Complement-mediated Cell Lysis Assay—Murine erythroleukemia (MEL) cells (a gift of Dr. Marilyn Farquhar, University of California, San Diego, CA) grown in DMEM containing 10% fetal bovine serum and 4 mM glutamine were rinsed three times with gelatin veronal buffer with Mg^{2+} and EGTA (GVB-MgEGTA, Sigma). Cells were resuspended in GVB-MgEGTA at a density of 5 × 10^6 cells/ml and incubated at 37 °C with different concentrations of normal human serum diluted in GVB-MgEGTA. After 2 h, cells were harvested for trypan blue exclusion assay by removing a 50-μl aliquot, incubating for 15 min in 0.4% trypan blue, and counting viable cells under an inverted microscope. The thiazolium blue assay was carried out at identical serum dilutions by incubating a 50-μl aliquot of the cell suspension in an equal volume of 1 mg/ml thiazolyl blue (MTT) for 45 min. Cell viability was determined spectrophotometrically by absorbance at 560 nm. Cells in GVB-MgEGTA buffer were preincubated with 10 μg of either rBSP or rOPN in 1 ml for 10 min at 37 °C. Normal human serum collected for good complement activity was then added at a dilution of 1:10 and the cells returned to 37 °C for 2 h and cell viability was determined by trypan blue exclusion and MTT assays.

For the assay of human cancer cell lines, loosely adherent MCF-7 cells were selected for by sequential growth in EMEM with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and 10% fetal bovine serum, while U-266 cells were cultured in RPMI 1640 medium containing 15% fetal bovine serum. Cells were collected by centrifugation and rinsed three times with GVB-MgEGTA buffer and subsequently treated exactly as for MEL cells, substituting guinea pig serum (Sigma) for human serum.

RESULTS

BSP Forms Complexes with a Serum-binding Protein—The status of BSP in human serum was studied using a number of well defined polyclonal antibodies against short peptides (26) and recombinant fragments that span the BSP molecule (30). Initially, we adapted an existing competitive ELISA for bone matrix-derived BSP (31) to determine levels of BSP in human serum, but were unable to detect any BSP. However, when 25-μl aliquots of normal human serum diluted 1:100 were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with a peptide-derived antibody against BSP, immunoreactive bands were readily apparent (Fig. 1*A*). Curiously, in the absence of reducing agent, the BSP immunoreactive band migrated with an estimated molecular mass of 250 kDa, while with reduction a migration position that corresponded authentic BSP (molecular mass ~80 kDa) was evident. BSP contains no cytseine residues hence the shift with reduction suggested that BSP in serum was tightly bound to another serum component.

BSP possesses a high degree of negative charge (pI < 4.0), therefore strong anion exchange HPLC was first employed to isolate the BSP complex. BSP from serum did not, however, bind to QAE resin unless it was previously subject to both heating and reduction (Fig. 1*B*). Immunoprecipitation was then used to further characterize the BSP complex in serum using six different polyclonal antibodies that span the BSP molecule. When the immunoprecipitates were subjected to SDS-PAGE resolution followed by Western blotting and detection of the BSP with a monoclonal antibody, only certain antibodies were able to immunoprecipitate BSP and even the best could precipitate only a small fraction of the total BSP in the serum. Immunoprecipitates generated by antibodies directed toward the carboxyl-terminal RGD-containing region of the molecule completely failed to immunoprecipitate BSP (Fig. 1, *C* and *D*). Taken together these results indicate that BSP is present in serum as a high molecular weight complex, which masks its negative charge, and that the RGD domain is not surface accessible.

Identification of the Serum-binding Protein—To identify the complex constituents, unreduced normal human serum was fractionated by size exclusion chromatography (SEC). The ma-

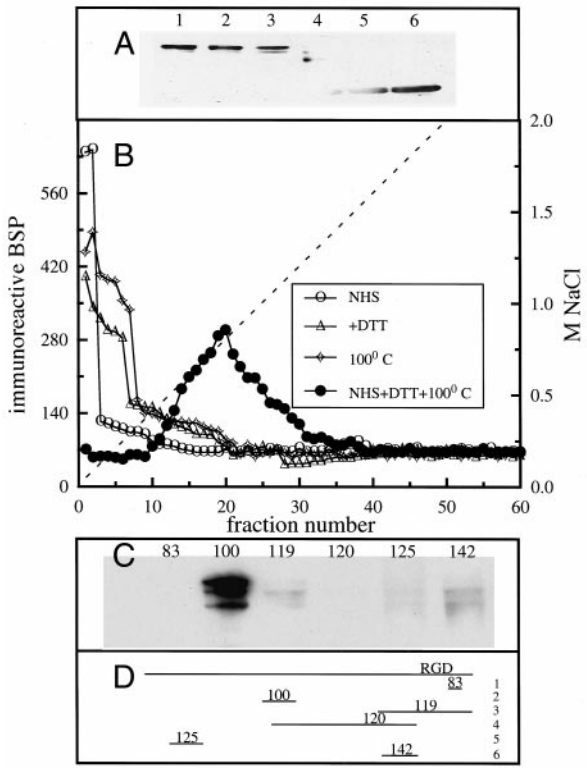

FIG. 1. **Bone sialoprotein in human serum.** *A*, normal human serum diluted 1:100 was subjected to SDS-PAGE in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of the reducing agent (2 mM dithiothreitol), the resolved proteins transferred to nitrocellulose, and the membrane was probed with LF-100 anti-BSP antibody. *B*, aliquots of normal human serum were analyzed by HPLC on a strong anion exchange column after 10 min at room temperature (○), in 2 mM dithiothreitol (*DTT*) (△), at 100 °C (◇), and in 2 mM dithiothreitol at 100 °C (●). *C*, immunoprecipitates from normal human serum incubated with LF-83 (*lane 1*), LF-100 (*lane 2*), LF-119 (*lane 3*), LF-120 (*lane 4*), LF-125 (*lane 5*), LF-142 (*lane 6*) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody, LFmAb-11, against BSP and immunoreactive material visualized by chemiluminescence. *D*, the approximate polypeptide sequences against which BSP antibodies were raised are indicated.

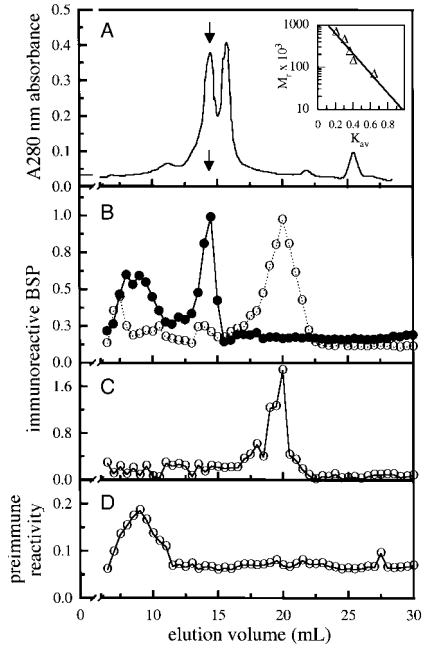

FIG. 2. **Identification of a serum binding factor.** Normal human serum diluted 1:100 was fractionated by SEC. *A*, protein elution positions were monitored by absorbance at 280 nm. Molecular weights were determined by calibration of the SEC column with known protein standards (*inset*). *B*, samples analyzed by SEC and direct BSP ELISA using LF-100 included 1:100 normal human serum (*solid circles*), and 1:100 normal human serum incubated with reducing agent and heat (*open circles* with *dotted line*). *C*, purified rBSP (2 ng) was fractionated by SEC and analyzed by direct BSP ELISA. *D*, an equal volume of 1:100 normal human serum was fractionated and screened with preimmune serum to determine nonspecific binding.

jority of immunoreactive BSP eluted as a single peak (Fig. 2, *arrow*). When aliquots of fractions from the SEC were transferred to 96-well microtiter plates and analyzed by direct ELISA for BSP, two peaks were apparent. Compared with protein standards, the BSP peak eluted at an estimated molecular mass of 250 ± 30 kDa. Analysis of the same elution profile using preimmune serum in place of anti-BSP polyclonal antibody in the direct ELISA yielded a single peak eluting in the excluded volume of the column, suggesting that immunoreactive material in the void of the anti-BSP profiles represents nonspecific immunoreactivity. Purified rBSP was found to elute as a single peak with a calculated molecular mass of 80 kDa. SEC resolution of a separate aliquot of the same normal human serum that had been incubated with reducing agent and heat to dissociate the binding complex yielded an immunoreactive profile upon direct ELISA analysis was identical to authentic BSP with a mass of 80 kDa.

The requirement of heating and reduction to disrupt the binding complex suggests that the BSP-binding serum component(s) possess multiple disulfide bonds and a stable structure. Subtracting the mass of BSP from the complex yields a mass estimate of 180 kDa for the other binding component(s). These observations taken together with the high content of sialic acid in BSP suggested complement Factor H as a potential binding complex constituent. Unreduced normal human serum was fractionated by SEC and subjected to Western blotting and detection with a monoclonal antibody against human complement Factor H. Immunoreactive bands were apparent in fractions corresponding to the elution position of the BSP complex (Fig. 3).

The association of BSP with complement Factor H was further investigated by reconstitution of the complex from purified components. Normal human serum incubated with biotinylated-rBSP yielded avidin-HRP immunoreactive and anti-BSP immunoreactive peaks whose elution position corresponded to that of the serum-BSP complex (Fig. 4). No free rBSP was measurable by either antibody detection system. Incubation of biotinylated-rBSP with purified human complement Factor H also yielded a SEC profile where a single peak corresponding to that of serum-BSP complex was detected by avidin-HRP. Treatment of reconstituted rBSP-Factor H complex with reducing agent and heat lead to a shift in the BSP immunoreactive peak to that of free BSP.

Thus, complement Factor H has been identified as a BSP-binding protein by immunoprecipitation, Western blotting, and immunoassay. Factor H, a molecular mass 150-kDa protein, is a key regulatory braking mechanism in normal and alternate complement-mediated cell lysis. It dissociates and thereby inactivates the assembled C3 convertase, serves as an essential accelerator of Factor I-mediated cleavage of C3b to iC3b, and sterically inhibits C5 binding to C3b (a prerequisite step for terminal pathway activation). The salient structural features of Factor H include 20 short consensus repeats that contain four cysteine residues forming two disulfide bonds per repeat. In addition, each short consensus repeat contains one conserved tryptophan residue per repeat and Factor H is known to interact with several sialic acid-containing proteins. BSP lacks any tryptophan, OPN has one, while Factor H contains a total of 25 tryptophan residues. Thus, the binding interaction be-

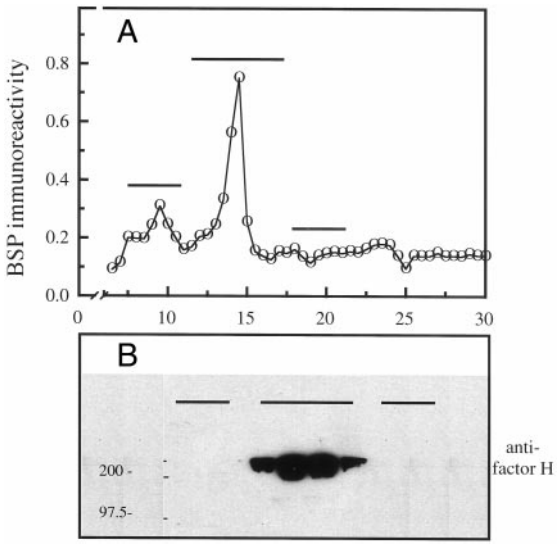

FIG. 3. **Identification of complement Factor H as the BSP serum-binding protein.** *A*, fractions from SEC analysis of unreduced normal human serum were resolved by 4–20% acrylamide gradient gel electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody against human complement Factor H. *B*, immunoreactive bands were visualized by chemiluminescent detection.

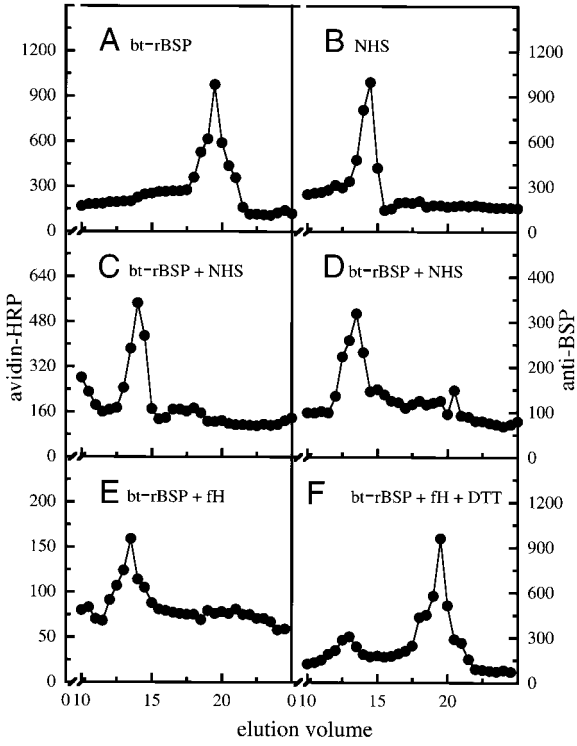

FIG. 4. **Reconstitution of the BSP-Factor H complex.** rBSP was isolated, biotinylated, and, after the indicated incubations, was subjected to SEC analysis. The incubations included: *A*, biotinylated rBSP alone; *B*, normal human serum (*NHS*) alone; *C* and *D*, NHS + biotinylated rBSP; *E*, biotinylated rBSP + purified complement Factor H; and *F*, biotinylated rBSP + purified complement Factor H treated with heating and reduction (*DTT*, dithiothreitol). Immunoreactive material was detected by either avidin-horseradish peroxidase (*A, C,* and *E*) or by direct ELISA for BSP (*B, D,* and *F*).

tween BSP or OPN and Factor H can be readily studied by intrinsic steady state fluorescence.

Titration of purified human complement Factor H with rBSP or rOPN was followed by excitation at 295 nm and monitoring emission between 300 and 450 nm. The emission profile of Factor H alone yields a peak at 347 nm (Fig. 5). The addition of

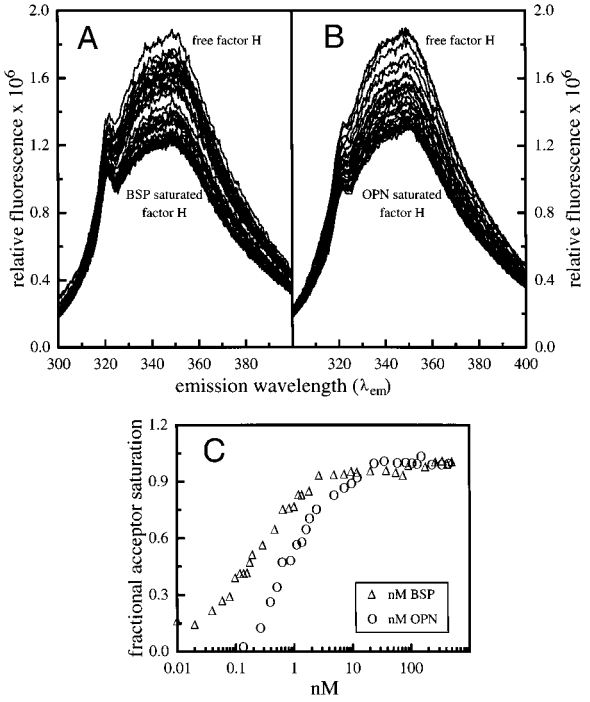

FIG. 5. **Fluorescence titration.** Intrinsic tryptophan fluorescence of tryptophan-rich Factor H was monitored by excitation at 295 nm and emission from 300 to 500 nm using a PTI series M fluorimeter. The initial Factor H concentration was 28 nM and rBSP (*A*) or rOPN (*B*) were added in nanomolar increments. Both Factor H as well as rBSP and rOPN were dissolved in Hank's balanced salt solution. *C*, binding curves were determined following calculation of fractional acceptor saturation.

rBSP or rOPN in nanomolar increments causes a relative fluorescent intensity quenching. Conversion of the fluorescent intensity titration into a binding curve by determining the fraction of binding sites occupied as the fractional change in fluorescence quenching at 347 nm yields a saturable binding curves (Fig. 5*C*). By steady state fluorescence, the binding of BSP and OPN by Factor H are saturable and possess a 1:1 stoichiometry, have binding constants in the nanomolar range, given the serum concentration of Factor H (~0.5 mg/ml), virtually all BSP and OPN in serum will be complexed with Factor H.

BSP and OPN Protect Tumor Cells from Alternate Complement-mediated Cell Lysis—Besides its key role in regulating complement and alternate complement activity, Factor H also appears to play a role when "hijacked" by invading organisms in enabling cellular evasion of complement. Pathogens such as *Streptococcus pyogenes* (32, 33), *Neisseria gonorrhoeae* (34–36), and *Echinococcus granulosus* (37, 38) bind Factor H to their cell surface and are resistant to complement-mediated cell lysis. In addition, molecular mimicry of Factor H where a pathogen makes a protein that is similar in sequence to Factor H to defend against attack by the host complement system has been described in vaccinia virus (39, 40), herpes simplex virus (41), and *Trypanosoma cruzi* (42–44). Within this context, it is interesting that *Staphylococcus aureus* isolated from patients with osteomyelitis has surface bound BSP (45). We have investigated whether BSP and OPN may play a similar role in tumor cell complement evasion by testing to see whether these small integrin-binding glycoproteins could promote tumor cell survival. Having identified the serum-binding component for BSP and OPN and bearing in mind the role of hijacked Factor H in pathogenic resistance to humoral surveillance, the ability of BSP and OPN to protect cells from complement activity was investigated. The first model system employed was a MEL cell line which when incubated with normal human serum can be readily

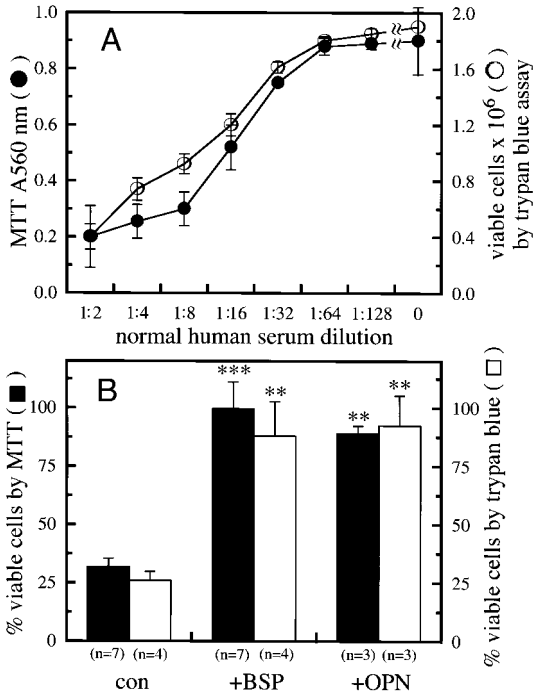

FIG. 6. **rBSP and rOPN protect MEL cells from complement-mediated lysis.** *A*, MEL cells were rinsed three times with GVB-MgEGTA buffer, resuspended in GVB-MgEGTA at a density of 5×10^6 cells/ml, and incubated at 37 °C with different concentrations of normal human serum. After 2 h, cells were harvested for trypan blue exclusion assay. The thiazolium blue assay was carried out at identical serum dilutions and cell viability was determined by absorbance at 560 nm. Each data point represents the average of three measurements. The *error bars* represent the standard deviation of the mean. *B*, MEL cells in GVB-MgEGTA buffer were incubated with 10 μg/ml either rBSP or rOPN for 10 min at 37 °C. Normal human serum was then added at a dilution of 1:10 and the cells returned to 37 °C, incubated for a further 2 h and cell viability was determined by trypan blue and MTT assays. Data from three to seven separate experiments, with treatment replicates in triplicate, was combined to yield mean values. Statistical significance was determined by analysis of variance. *Error bars* represent the S.E. of the mean. n = number of experiments combined; **, $p \leq 0.01$; ***, $p \leq 0.001$.

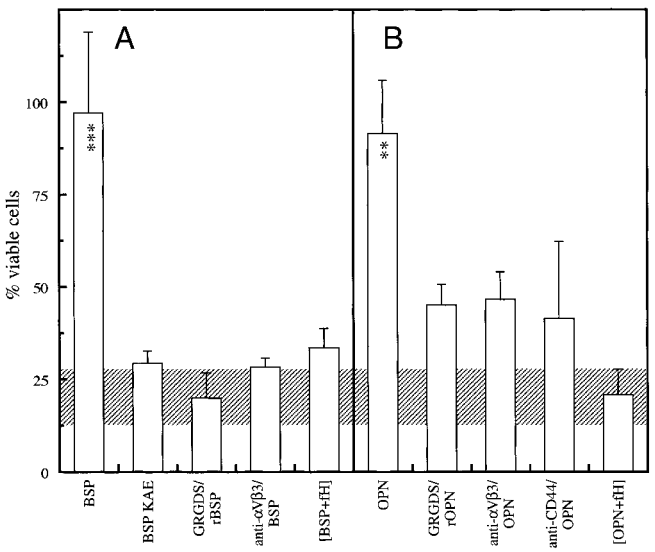

FIG. 7. **Integrin and CD44 involvement in protection from complement-mediated lysis.** *A*, MEL cells prepared as described in the legend to Fig. 6 were treated with normal rBSP (10 μg/ml), rBSP whose RGD sequence had been mutated to KAE (10 μg/ml), and either GRGDS peptide (400 μM) followed by rBSP or an $\alpha_V\beta_3$ antibody (1:4000) followed by rBSP for 10 min prior to the addition of normal human serum. The cells were then incubated for 10 min after which cell viability determined using the MTT assay. *B*, a cohort of MEL cells were pretreated with rOPN (10 μg/ml) alone, GRGDS peptide followed by rOPN, the $\alpha_V\beta_3$ antibody followed by rOPN, or an anti-CD44 antibody (Chemicon, Co.) followed by rOPN, or hyaluronan followed by rOPN. Cells were then treated with normal human serum and viability assayed as described in the legend to Fig. 6. Treatment of MEL cells with a pre-formed complex of either BSP-Factor H (*BSP+fH*) or OPN-Factor H (*OPN+fH*) abolished the protection from complement-mediated lysis. Percent cell viability was determined was determined using A_{560} absorbance values of various conditions and a control where no serum had been added (100% viable). The *cross-hatched* region represents that range of values observed when normal human serum (1:10) alone was added (maximal cell death). The data represents the mean and S.E. for three separate experiments. Statistical significance was determined by analysis of variance. **, $p \leq 0.01$; ***, $p \leq 0.001$.

assayed for ACP-mediated cell lysis (46). Cell survival was measured by both trypan blue dye exclusion and thiazolyl blue (MTT) reduction by living mitochondria. Titration with dilutions of normal human serum and time courses were carried out to define optimal incubation conditions. At 1:10 dilution, human serum totally lysed the MEL cells as measured by both assay systems (Fig. 6*A*). The addition of purified recombinant BSP to MEL cells followed by treatment with normal human serum completely protected the cells from lysis (Fig. 6*B*). Treatment of MEL cells with recombinant OPN also conferred protection from ACP-mediated cell lysis (Fig. 6*B*). The protection of MEL cells from alternate complement pathway-mediated cell lysis by both BSP and OPN exhibited dose responses (data not shown).

The mechanism of protection from complement-mediated lysis was investigated. Preincubation of MEL cells with rBSP whose RGD sequence had been mutated to KAE completely removed the protective affects of this protein (Fig. 7). Furthermore, preincubation of MEL cells with either GRGDS peptide or an $\alpha_V\beta_3$ antibody (which blocks that integrins binding activity) negated the protective effect of rBSP. Up-regulation of the $\alpha_V\beta_3$ integrin has also been shown to correlate with invasive potential (47). For OPN, pretreatment with GRGDS peptide or the $\alpha_V\beta_3$ antibody reduced cell survival, although the magnitude of reduction was not as great as that seen for BSP (Fig. 7*B*). OPN has multiple potential receptors including $\alpha_V\beta_1$, β_3, β_5-containing integrin receptors as well as CD44, a receptor implicated in attachment, homing, and aggregation of lymphocytes as well as neoplastic cells. Pretreatment of MEL cells with hyaluronan, a natural ligand for CD44 (48), as well as with an anti-CD44 antibody also reduced the protective effect of added rOPN (Fig. 7). Treatment of MEL cells with a pre-formed complex of either rBSP-Factor H or rOPN-Factor H abolished the protection from complement-mediated lysis. For BSP, this is consistent with immunoprecipitation data that indicates that the RGD moiety is inaccessible in the solution phase Factor H complex. These data suggest that both of these proteins may be entirely masked by Factor H shortly after being secreted by a cell.

To verify that this protective effect of BSP and OPN might be operative in human cancer cells, MCF-7 breast cancer cells selected for nonadherent growth and U-266 myeloma cells were used in the alternate complement pathway cell lysis assay with guinea pig serum as the source of complement activity. Both cell types exhibited enhanced survival and protection from complement-mediated cell lysis when rBSP or rOPN were present (Fig. 8). Increasing concentration of guinea pig serum lead to decreasing cell viability, while the pretreatment with either 10 μg/ml rBSP or rOPN resulted in increased cell viability.

DISCUSSION

The survival of trophoblasts and neoplasms requires resistance to immunologic recognition and subsequent attack by host. Host surveillance pathways include B and T cell lymphocytes and macrophages in immune response as well as complement-mediated attack and lysis. Immune transparency is aided

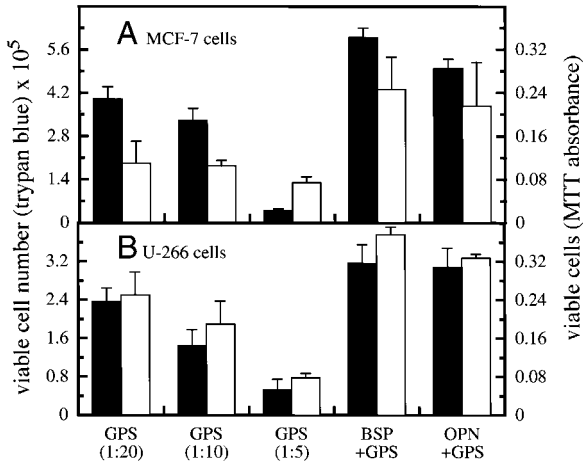

FIG. 8. **Human cancer cell lines demonstrate alternate complement susceptibility, and sialoproteins confer protection from lytic activity.** Human cancer cell lines, MCF-7 (*A*) and U-266 (*B*) cells were rinsed three times with GVB-MgEGTA buffer and subsequently treated exactly as for MEL cell complement-mediated cell lysis, substituting complement active guinea pig serum (*GPS*) for human serum. Three different dilutions of GPS were assayed. For cultures pretreated with 10 μg/ml BSP or OPN, the dilution of GPS used was 1:5. Cell viability was determined by both trypan blue exclusion (*solid bar*) and MTT (*open bar*) assay. The *bars* represent the mean values of triplicate samples and the *error bars* are the standard deviation values.

and abetted in trophoblasts by the lack of expression of major histocompatibility complex antigens which present peptides to CD8+ cytotoxic T cells (49); the expression of nonclassical, nonpolymorphic HLA-G which inhibits natural killer cells (50, 51); the dominance of the type 2 T-helper cells over type 1 T-helper cells (52); the immunosuppressive effect of induced prostaglandin E_2 (53); and induction of apoptosis in Fas-bearing activated lymphocytes by placental expression of Fas ligand (54, 55). Similarly, for neoplasms, continued subversion of host surveillance may involve a deficiency or lack of expression of major histocompatibility complex antigens (56, 57); dysregulation between HLA class I antigen expression and natural killer cell activity (58); an expansion in type 2 T-helper cells and a malfunction in type 1 T-helper cells (59, 60); enhanced production of prostaglandin E_2 (61, 62); and neoplastic expression of Fas-ligand (63, 64). However, host surveillance mechanisms also include the complement system (65).

The complement system found in the blood of mammals is composed of about 26 proteins that combine with antibodies or cell surfaces as part of host humoral surveillance. Complement plays a role in inflammation, immune adherence, opsonization, viral neutralization, cell lysis, and localization of antigen (66). The complement system can be activated via two distinct pathways: the classical (antibody initiated) pathway and the alternate pathway. The alternate pathway is the more ancient surveillance system and consists of a series of humoral protease activation cascades that result in, among other things, the formation of membrane pores that depolarize and kill the cell (67, 68). Virtually all cells are subject to low levels of this attack all the time, but only cells whose surfaces cannot quickly inactivate the early steps of the cascade, C3b convertase formation, are killed. Cells that are destined to become transformed and escape the complement system may up-regulate genes that help to control this aspect of immune surveillance (69–72). The ability to bind and use the natural ACP inhibitory actions of Factor H would be one method of escape. Factor H inhibits the production of C3b by inhibiting the binding of Factor B to membrane-bound C3b, thereby preventing cleavage of B to Bb and production of the C3 convertase, C3b2b. Factor H also accelerates Factor I-mediated cleavage of C3b and sterically inhibits C5 binding to C3b. Both classical and alternate complement pathways are being investigated as potential therapeutic targets in cancer (65, 69–73). Within this context it is of note that an alternatively spliced form of Factor H, Factor H-related protein, has recently been shown to be a biomarker for bladder cancer. (74–78).

Recent observations have shown that BSP and OPN are expressed by malignant tissue. BSP is expressed in primary breast cancers (4, 8, 9, 79), prostate cancer (19), lung cancer (10), thyroid cancer (11), malignant bone disease (80), and neoplastic odonotoblasts (81). In addition, BSP peptides are potent inhibitors of breast cancer cell adhesion to bone (82, 83). OPN is expressed in breast cancer (4, 7, 16, 23), as well as in prostate cancer (21), thyroid cancer (84), skin cancer (85), and several other types of cancer (6, 86, 87).

Trophoblasts and metastasizing cancer cells are exposed to the highest levels of complement because they are in direct contact with host blood. To survive, trophoblasts and neoplasms need to directly control the complement activity on their surfaces and thus aid their escape not only from direct lysis but also from being opsonized by the alternative complement pathway. Additionally, macrophages, the effector cells in immune surveillance, are activated by ACP (88). Thus, agents that inhibit or down-regulate complement decrease both the direct lysis pathway of complement as well as macrophage activation and thereby promote tumor survival. The expression of OPN and BSP in tumor cells could provide such a selective advantage for survival via (*a*) initial binding to $\alpha_V\beta_3$ integrins (both) or CD44 (OPN) on the cell surface, (*b*) sequestration of Factor H to the cell surface, and (*c*) Factor H-mediated inhibition of complement-mediated cell lysis and opsonization. *In vitro* experiments using a murine erythroleukemia cell line have yielded consistent results where OPN or BSP is protective against human complement-mediated cell lysis. They have also been shown to protect two human cancer cell lines from attack by guinea pig complement.

We have shown that Factor H has a high affinity for both BSP and OPN and that if the complex between these glycoproteins and Factor H occurs before they can bind to their cell surface receptors, then the ability to protect from complement-mediated attack is lost. Because Factor H is found at 0.5 mg/ml in the serum and lower but significant levels in most tissue spaces, this suggests that the ACP-protective pathways of these two proteins are limited to autocrine or paracrine distances from their sites of secretion. Furthermore, because virtually the entire length of BSP (and apparently as much of OPN) is buried within the complex and inaccessible to antisera and other binding proteins, any other functions that these proteins may serve will likely also be extremely limited in their functional ranges.

The induction of specific genes not usually expressed by a given differentiated cell type is one of the hallmarks of neoplastic transformation. The switching on of these genes can be part of a generalized alteration in phenotypic expression where the *de novo* production of the gene product is not a necessary part of the neoplastic process (there is no gain of function by its expression). It is also possible that the expression of these cancer-associated genes confers a gain of function or selective survival advantage to the neoplasm. Using MEL cells as well as human breast cancer and myeloma cells and recombinant BSP and OPN, we have found that, following interaction with a specific receptors on the cell surface, BSP and OPN sequester Factor H to the cell surface and that this interaction quenches alternate complement-mediated cell lysis by normal human serum. These results suggest a shared mechanism between trophoblasts and neoplasms for evading host surveillance through "molecular cloaking" via Factor H sequestration and dampening of the complement-mediated cell lysis and opsonization.

REFERENCES

1. Omigbodun, A., Daiter, E., Walinsky, D., Fisher, L., Young, M., Hoyer, J., and Coutifaris, C. (1995) *Ann. N. Y. Acad. Sci.* **760,** 346–349
2. Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D., and Robey, P. G. (1991) *Calcif. Tissue Int.* **49,** 421–426
3. Craig, A. M., Bowden, G. T., Chambers, A. F., Spearman, M. A., Greenberg, A. H., Wright, J. A., McLeod, M., and Denhardt, D. T. (1990) *Int. J. Cancer* **46,** 133–137
4. Gillespie, M. T., Thomas, R. J., Pu, Z. Y., Zhou, H., Martin, T. J., and Findlay, D. M. (1997) *Int. J. Cancer* **73,** 812–815
5. Hirota, S., Nakajima, Y., Yoshimine, T., Kohri, K., Nomura, S., Taneda, M., Hayakawa, T., and Kitamura, Y. (1995) *J. Neuropathol. Exp. Neurol.* **54,** 698–703
6. Senger, D. R., Perruzzi, C. A., and Papadopoulos, A. (1989) *Anti-cancer Res.* **9,** 1291–1299
7. Sung, V., Gilles, C., Murray, A., Clarke, R., Aaron, A. D., Azumi, N., and Thompson, E. W. (1998) *Exp. Cell Res.* **241,** 273–284
8. Bellahcene, A., Merville, M. P., and Castronovo, V. (1994) *Cancer Res.* **54,** 2823–2826
9. Bellahcene, A., Menard, S., Bufalino, R., Moreau, L., and Castronovo, V. (1996) *Int. J. Cancer* **69,** 350–353
10. Bellahcene, A., Maloujahmoum, N., Fisher, L. W., Pastorino, H., Tagliabue, E., Menard, S., and Castronovo, V. (1997) *Calcif. Tissue Int.* **61,** 183–188
11. Bellahcene, A., Albert, V., Pollina, L., Basolo, F., Fisher, L. W., and Castronovo, V. (1998) *Thyroid* **8,** 637–641
12. Fisher, L. W., Hawkins, G. R., Tuross, N., and Termine, J. D. (1987) *J. Biol. Chem.* **262,** 9702–9708
13. Fisher, L. W., McBride, O. W., Termine, J. D., and Young, M. F. (1990) *J. Biol. Chem.* **265,** 2347–2351
14. Oldberg, A., Franzen, A., and Heinegard, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8819–8823
15. Castronovo, V., and Bellahcene, A. (1998) *Int. J. Oncol.* **12,** 305–308
16. Bellahcene, A., and Castronovo, V. (1997) *Bull. Cancer* **84,** 17–24
17. Sung, V., Stubbs, J. T., 3rd, Fisher, L., Aaron, A. D., and Thompson, E. W. (1998) *J. Cell. Physiol.* **176,** 482–494
18. Bellahcene, A., Kroll, M., Liebens, F., and Castronovo, V. (1996) *J. Bone Miner. Res.* **11,** 665–670
19. Waltregny, D., Bellahcene, A., Van Riet, I., Fisher, L. W., Young, M., Fernandez, P., Dewe, W., de Leval, J., and Castronovo, V. (1998) *J. Natl. Cancer Inst.* **90,** 1000–1008
20. Chambers, A. F., Wilson, S. M., Kerkvliet, N., O'Malley, F. P., Harris, J. F., and Casson, A. G. (1996) *Lung Cancer* **15,** 311–323
21. Koeneman, K. S., Yeung, F., and Chung, L. W. (1999) *Prostate* **39,** 246–261
22. Oates, A. J., Barraclough, R., and Rudland, P. S. (1997) *Invasion & Metastasis* **17,** 1–15
23. Tuck, A. B., O'Malley, F. P., Singhal, H., Harris, J. F., Tonkin, K. S., Kerkvliet, N., Saad, Z., Doig, G. S., and Chambers, A. F. (1998) *Int. J. Cancer* **79,** 502–508
24. Ue, T., Yokozaki, H., Kitadai, Y., Yamamoto, S., Yasui, W., Ishikawa, T., and Tahara, E. (1998) *Int. J. Cancer* **79,** 127–132
25. Tiniakos, D. G., Yu, H., and Liapis, H. (1998) *Hum. Pathol.* **29,** 1250–1254
26. Fisher, L. W., Stubbs, J. T., 3rd, and Young, M. F. (1995) *Acta Orthop. Scand. Suppl.* **266,** 61–65
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
28. Young, M. F., Kerr, J. M., Termine, J. D., Wewer, U. M., Wang, M. G., McBride, O. W., and Fisher, L. W. (1990) *Genomics* **7,** 491–502
29. Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) *Methods Cell Biol.* **43,** 161–189
30. Stubbs, J. T., 3rd, Mintz, K. P., Eanes, E. D., Torchia, D. A., and Fisher, L. W. (1997) *J. Bone Miner. Res.* **12,** 1210–1222
31. Vetter, U., Fisher, L. W., Mintz, K. P., Kopp, J. B., Tuross, N., Termine, J. D., and Robey, P. G. (1991) *J. Bone Miner. Res.* **6,** 501–505
32. Blackmore, T. K., Fischetti, V. A., Sadlon, T. A., Ward, H. M., and Gordon, D. L. (1998) *Infect. Immun.* **66,** 1427–1431
33. Kotarsky, H., Hellwage, J., Johnsson, E., Skerka, C., Svensson, H. G., Lindahl, G., Sjobring, U., and Zipfel, P. F. (1998) *J. Immunol.* **160,** 3349–3354
34. Jarvis, G. A., and Vedros, N. A. (1987) *Infect. Immun.* **55,** 174–180
35. Ram, S., McQuillen, D. P., Gulati, S., Elkins, C., Pangburn, M. K., and Rice, P. A. (1998) *J. Exp. Med.* **188,** 671–680
36. Ram, S., Sharma, A. K., Simpson, S. D., Gulati, S., McQuillen, D. P., Pangburn, M. K., and Rice, P. A. (1998) *J. Exp. Med.* **187,** 743–752
37. Diaz, A., Ferreira, A. M., and Nieto, A. (1995) *Exp. Parasitol.* **80,** 473–482
38. Diaz, A., Ferreira, A., and Sim, R. B. (1997) *J. Immunol.* **158,** 3779–3786
39. Wiles, A. P., Shaw, G., Bright, J., Perczel, A., Campbell, I. D., and Barlow, P. N. (1997) *J. Mol. Biol.* **272,** 253–265
40. Isaacs, S. N., Kotwal, G. J., and Moss, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 628–632
41. Huemer, H. P., Wang, Y., Garred, P., Koistinen, V., and Oppermann, S. (1993) *Immunology* **79,** 639–647
42. Donelson, J. E., Hill, K. L., and El-Sayed, N. M. (1998) *Mol. Biochem. Parasitol.* **91,** 51–66
43. Hall, B. F., and Joiner, K. A. (1993) *J. Eukaryot. Microbiol.* **40,** 207–213
44. Kipnis, T. L., and da Silva, W. D. (1989) *Braz. J. Med. Biol. Res.* **22,** 1–16
45. Ryden, C., Yacoub, A. I., Maxe, I., Heinegard, D., Oldberg, A., Franzen, A., Ljungh, A., and Rubin, K. (1989) *Eur. J. Biochem.* **184,** 331–336
46. Shi, W. X., Chammas, R., Varki, N. M., Powell, L., and Varki, A. (1996) *J. Biol. Chem.* **271,** 31526–31532
47. Varner, J. A., and Cheresh, D. A. (1996) *Curr. Opin. Cell Biol.* **8,** 724–730
48. Culty, M., Miyake, K., Kincade, P. W., Sikorski, E., Butcher, E. C., Underhill, C., and Silorski, E. (1990) *J. Cell Biol.* **111,** 2765–2774
49. Faulk, W. P., and Temple, A. (1976) *Nature* **262,** 799–802
50. Kovats, S., Main, E. K., Librach, C., Stubblebine, M., Fisher, S. J., and DeMars, R. (1990) *Science* **248,** 220–223
51. Munz, C., Holmes, N., King, A., Loke, Y. W., Colonna, M., Schild, H., and Rammensee, H. G. (1997) *J. Exp. Med.* **185,** 385–391
52. Wegmann, T. G., Lin, H., Guilbert, L., and Mosmann, T. R. (1993) *Immunol. Today* **14,** 353–356
53. Thorburn, G. D. (1991) *Reprod. Fertil. Dev.* **3,** 277–294
54. Kauma, S. W., Huff, T. F., Hayes, N., and Nilkaeo, A. (1999) *J. Clin. Endocrinol. Metab.* **84,** 2188–2194
55. Coumans, B., Thellin, O., Zorzi, W., Melot, F., Bougoussa, M., Melen, L., Zorzi, D., Hennen, G., Igout, A., and Heinen, E. (1999) *J. Immunol. Methods* **224,** 185–196
56. Smith, M. E., Marsh, S. G., Bodmer, J. G., Gelsthorpe, K., and Bodmer, W. F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5557–5561
57. Moller, P., and Hammerling, G. J. (1992) *Cancer Surv.* **13,** 101–127
58. Pena, J., Alonso, C., Solana, R., Serrano, R., Carracedo, J., and Ramirez, R. (1990) *Eur. J. Immunol.* **20,** 2445–2448
59. Pellegrini, P., Berghella, A. M., Del Beato, T., Cicia, S., Adorno, D., and Casciani, C. U. (1996) *Cancer Immunol. Immunother.* **42,** 1–8,
60. Roussel, E., Gingras, M. C., Grimm, E. A., Bruner, J. M., and Moser, R. P. (1996) *Clin. Exp. Immunol.* **105,** 344–352
61. Husby, G., Strickland, R. G., Rigler, G. L., Peake, G. T., and Williams, R. C., Jr. (1977) *Cancer* **40,** 1629–1642
62. Cummings, K. B., and Robertson, R. P. (1977) *J. Urol.* **118,** 720–723
63. Gutierrez, L. S., Eliza, M., Niven-Fairchild, T., Naftolin, F., and Mor, G. (1999) *Breast Cancer Res. Treat.* **54,** 245–253
64. Strand, S., and Galle, P. R. (1998) *Mol. Med. Today* **4,** 63–68
65. Cooper, P. D. (1985) in *Advances in Immunity and Cancer Therapy* (Ray, P. K., ed) Vol. 1, pp. 127–166, Springer-Verlag, New York
66. Volanakis, J. E. (1998) in *The Human Complement System in Health and Disease* (Volankis, J. E., and Frank, M. M., eds) pp. 9–32, Marcel Dekker, Inc., New York
67. Krushkal, J., Kemper, C., and Gigli, I. (1998) *J. Mol. Evol.* **47,** 625–630
68. Meri, S., and Jarva, H. (1998) *Vox Sang* **74,** Suppl. 2, 291–302
69. Simpson, K. L., Jones, A., Norman, S., and Holmes, C. H. (1997) *Am. J. Pathol.* **151,** 1455–1467
70. Schmitt, C. A., Schwaeble, W., Wittig, B. M., Meyer zum Buschenfelde, K. H., and Dippold, W. G. (1999) *Eur. J. Cancer* **35,** 117–124
71. Spendlove, I., Li, L., Carmichael, J., and Durrant, L. G. (1999) *Cancer Res.* **59,** 2282–2286
72. Yu, J., Caragine, T., Chen, S., Morgan, B. P., Frey, A. B., and Tomlinson, S. (1999) *Clin. Exp. Immunol.* **115,** 13–18
73. Ross, G. D., Vetvicka, V., Yan, J., Xia, Y., and Vetvickova, J. (1999) *Immunopharmacology* **42,** 61–74
74. Irani, J., Desgrandchamps, F., Millet, C., Toubert, M. E., Bon, D., Aubert, J., and Le Duc, A. (1999) *Eur. Urol.* **35,** 89–92
75. Kinders, R., Jones, T., Root, R., Bruce, C., Murchison, H., Corey, M., Williams, L., Enfield, D., and Hass, G. M. (1998) *Clin Cancer Res.* **4,** 2511–2520
76. Nasuti, J. F., Gomella, L. G., Ismial, M., and Bibbo, M. (1999) *Diagn. Cytopathol.* **21,** 27–29
77. Raitanen, M. P., and Tammela, T. L. (1999) *Scand. J. Urol. Nephrol.* **33,** 234–236
78. Thomas, L., Leyh, H., Marberger, M., Bombardieri, E., Bassi, P., Pagano, F., Pansadoro, V., Sternberg, C. N., Boccon-Gibod, L., Ravery, V., Le Guludec, D., Meulemans, A., Conort, P., and Ishak, L. (1999) *Clin. Chem.* **45,** 472–477
79. Bellahcene, A., Antoine, N., Clausse, N., Tagliabue, E., Fisher, L. W., Kerr, J. M., Jares, P., and Castronovo, V. (1996) *Lab. Invest.* **75,** 203–210
80. Seibel, M. J., Woitge, H. W., Pecherstorfer, M., Karmatschek, M., Horn, E., Ludwig, H., Armbruster, F. P., and Ziegler, R. (1996) *J. Clin. Endocrinol. Metab.* **81,** 3289–3294
81. Chen, J., Aufdemorte, T. B., Jiang, H., Liu, A. R., Zhang, W., and Thomas, H. F. (1998) *Histochem. J.* **30,** 1–6
82. van der Pluijm, G., Vloedgraven, H. J., Ivanov, B., Robey, F. A., Grzesik, W. J., Robey, P. G., Papapoulos, S. E., and Lowik, C. W. (1996) *Cancer Res.* **56,** 1948–1955
83. van der Pluijm, Vloedgraven, H., Papapoulos, S., Lowick, C., Grzesik, W., Kerr, J., and Robey, P. G. (1997) *Lab. Invest.* **77,** 665–675
84. Tunio, G. M., Hirota, S., Nomura, S., and Kitamura, Y. (1998) *Arch. Pathol. Lab. Med.* **122,** 1087–1090
85. Wright, G. L., Jr., Huang, C. L., Lipford, G., Beckett, M. L., Liang, H. M., Haley, C., Newhall, K., and Morningstar, M. (1990) *Int. J. Cancer* **46,** 39–49
86. Senger, D. R., Perruzzi, C. A., Papadopoulos, A., and Tenen, D. G. (1989) *Biochim. Biophys. Acta* **996,** 43–48
87. Chambers, A. F., Behrend, E. I., Wilson, S. M., and Denhardt, D. T. (1992) *Anticancer Res.* **12,** 43–47
88. Mevorach, D., Mascarenhas, J. O., Gershov, D., and Elkon, K. B. (1998) *J. Exp. Med.* **188,** 2313–2320